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Zoledronic Acid Activates the DNA S-Phase Checkpoint and Induces Osteosarcoma Cell Death Characterized by Apoptosis-Inducing Factor and Endonuclease-G Translocation Independently of p53 and Retinoblastoma Status

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ABSTRACT

The molecular mechanisms responsible for the cellular effects of the nitrogen-containing bisphosphonate zoledronic acid (Zol) were assessed on several osteosarcoma cell lines differing in their p53 and retinoblastoma (Rb) status. Zol inhibited cell proliferation and increased atypical apoptosis. The Zol effects on proliferation were due to cell cycle arrest in S and G_2/M phases subsequent to the activation of the intra-S DNA damage checkpoint with an increase in P-ATR, P-chk1, Wee1, and P-cdc2 levels and a decrease in cdc25c, regardless of the p53 and Rb status. In addition, the atypic apoptosis induced by Zol was independent of caspase activation, and it was characterized by nuclear alterations, increased Bax expression, and reduced Bcl-2 level. Furthermore, mitochondrial permeability was up-regulated by Zol independently of p53 in association with the translocation of apoptosis-inducing factor (AIF) and endonuclease-G (EndoG). Zol also disturbed cytoskeletal organization and cell junctions and inhibited cell migration and phosphorylation of focal adhesion kinases. The main difficulty encountered in treating cancer relates to mutations in key genes such as p53, Rb, or proteins affecting caspase signaling carried by many tumor cells. We have demonstrated for the first time that zoledronic acid activated the DNA damage S-phase checkpoint and the mitochondrial pathway via AIF and EndoG translocation, and it inhibited cell proliferation and induced cell death, bypassing these potentials mutations. Therefore, zoledronic acid may be considered as an effective therapeutic agent in clinical trials of osteosarcoma in which mutation for p53 and Rb very often occur, and where current treatment with traditional chemotherapeutic agents is ineffective.

Bisphosphonates (BPs) are stable synthetic analogs of the naturally occurring pyrophosphate (Heymann et al., 2004). Different side chains can be added to the central carbon atom, thus producing a range of BPs with varying clinical activity and potency (Rogers et al., 2000). Therefore, BPs can be grouped into two classes of non–nitrogen-containing and nitrogen-containing BPs. The clinical use of bisphosphonates has increased dramatically during the past decade. The most common indicator for the use of these compounds is osteoporosis, but their use has rapidly emerged in osteolytic bone diseases characterized by enhanced bone resorption (e.g., Paget's disease and hypercalcemia of malignancy). Indeed, BPs are currently the most effective class of antiresorptive drugs available, and their first targets identified were osteoclasts. Due to the high tropism of BPs for hydroxyapatite in bone and the ability of osteoclasts to release bone-bound bisphosphonate, a direct effect on mature osteoclasts seems to be the most important mechanism of action. BPs can be grouped into two classes of non–nitrogen-containing and nitrogen-containing BPs. The BPs that lack a nitrogen atom and are most closely related to pyrophosphate (such as clodronate, etidronate, and tiludronate) are metabolized intracellularly to cytotoxic analogs of ATP that reduce osteoclast

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ABBREVIATIONS: BP, bisphosphonate; FPP, farnesyl diphosphate; Zol, zoledrenic acid; Rb, retinoblastoma; AIF, apoptosis-inducing factor; EndoG, endonuclease G; XTT, sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; FAK, focal adhesion kinase; RGD, Arg-Gly-Asp; PBS, phosphate-buffered saline; GGO, geranylgeraniol; cdk, cyclin-dependent kinase; Chk, checkpoint kinase; PARP1, poly(ADP-ribose) polymerase 1; ATR, ataxia-telangiectasia, mutated and Rad 3-related.

survival (Rogers et al., 2000). In contrast, the more potent nitrogen-containing bisphosphonates (such as pamidronate, alendronate, risedronate, ibandronate, and zoledronate) induce apoptosis in osteoclasts by inhibiting enzymes of the mevalonate pathway, mainly farnesyl diphosphate synthase (FPP) (Gibbs and Oliff, 1997). Inhibition of this enzyme in osteoclasts prevents the biosynthesis of cholesterol and isoprenoid lipids (FPP and geranylgeraniol diphosphate) that are essential for the post-translational farnesylation and geranylgeranylation of small GTPase signaling proteins. Loss of bone-resorptive activity and induction of osteoclast apoptosis is due primarily to loss of geranylgeranylated small GTPases (Coxon et al., 2000).

In addition to their potent antiosteoclastic effects, recent preclinical studies have shown that *N*-BPs induce apoptosis of cancer cells from several origins, including myeloma, breast, prostate carcinoma, and osteosarcoma cell lines (Mackie et al., 2001; Sonnemann et al., 2001). Recent clinical trials in patients with bone metastases or multiple myeloma demonstrated that zoledronic acid (Zol) was safe and well tolerated at the approved dose of 4 mg i.v. every 3 to 4 weeks (Heymann et al., 2004). Moreover, growing preclinical evidence shows that Zol also exhibits direct antitumor activity. Evdokiou et al. (2003) reported that Zol reduced the cell number of different human osteogenic sarcoma cell lines by a mechanism resembling anoikis. More recently, Kubista et al. (2006) confirmed these findings and showed that Zol regulates the cyclin and p27 expression. In this work, Zol induced cell death by a typical apoptotic pathway associated with nuclear fragmentation and Annexin-V staining. These authors suggested that the treatment of osteosarcoma cells by Zol might lead to mitotic catastrophes and consequently to the induction of cell death (Kubista et al., 2006). We also reported recently the enhancement of tumor regression and tissue repair when Zol is combined with ifosfamide in rat osteosarcoma (Heymann et al., 2005). Furthermore, Zol suppresses lung metastases and prolongs overall survival of osteosarcoma-bearing mice (Ory et al., 2005). However, the overall effects on osteosarcoma cells seem to be mediated via diverse and unclear pathways, such as apoptosis, proliferation, and metabolic events that need be clarified.

Because Zol represents a potential novel antineoplasic agent for the therapy of osteosarcoma, the present study investigated the cellular effects of Zol on several osteosarcoma cell lines possessing different p53 and Rb status, in particular OSRGA (p53 and Rb wild type), MG3 (p53 mutated and Rb wild type), and SaOS2 (p53 null and Rb-defective). We provide evidence that Zol exerts dual effects on osteosarcoma cell proliferation: high doses of Zol exert antiproliferative effects on osteosarcoma cells, resulting in their cell cycle arrest in S and G_2/M phases through the control of the intra-S DNA checkpoint. In contrast, low doses of Zol promote osteosarcoma cell proliferation through the control of the G_1/S DNA checkpoint. Furthermore, we report that Zol induces atypic apoptosis independently of caspase activation but involves the mitochondrial pathway, in particular AIF and EndoG translocation. Overall results demonstrate selective and original antitumor effects of Zol on several osteosarcoma cell lines independently of their p53 and Rb status, thus allowing these molecules to be considered as potential therapeutic agents in clinical trials of tumor bone pathologies, regardless of the p53 and Rb status of the patients.

Materials and Methods

Cells and Culture Conditions. The rat osteosarcoma OSRGA cell line was initially established from a radioinduced osteosarcoma (Klein et al., 1977; Thiéry et al., 1982). The rat ROS17/2.8 osteosarcoma cell line was kindly provided by Prof. H. J. Donahue (The Pennsylvania State University, University Park, PA), and the human MG63, SaOS2, U2OS, and MNNG-HOS cell lines were purchased from American Type Culture Collection (Manassas, VA). These cell lines were cultured in Dulbecco's modified Eagle's medium (Cambrex Bio Science Verriers S.p.r.l., Verviers, Belgium) supplemented with 5% fetal calf serum (Hyclone Laboratories, Brebières, France) and 2 mM L-glutamine (Cambrex Bio Science Verriers S.p.r.l.). Primary rat and human osteoblasts were isolated from bone explants and cultured in RPMI 1640 medium (Cambrex Bio Science Verriers S.p.r.l.) supplemented with 10% fetal calf serum and antibiotic mixture (100 IU/ml penicillin and 100 μg/ml streptomycin).

Cell Growth and Viability. Cell growth and viability were determined by a cell proliferation reagent assay kit using sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro-)benzene sulfonic acid hydrate (XTT) (Roche Molecular Biochemicals, Mannheim, Germany). Two thousand cells per well were plated into 96-well plates and cultured for 72 h in culture medium in the presence or absence of 10^{-12} to 10^{-4} M Zol and/or in the presence or absence of 1 and 10 mM RGD peptide (Arg-Gly-Asp consensus sequence for integrin interaction) (Sigma, St.-Quentin-Fallavier, France) diluted in the culture medium. Zol was provided by Pharma Novartis AG (Basel, Switzerland) as the disodium hydrate form. Additional experiments were performed in presence of geranylgeraniol (GGO) (Sigma). After the culture period, XTT reagent was added to each well and incubated for 5 h at 37°C; absorbance was then read at 490 nm using a 96-multiwell microplate reader. Cell viability was also assessed by trypan blue exclusion, and alive and dead cells were manually counted from trypsinized and floating cells. A minimum of 100 cells were counted in each culture condition. Cell death was also monitored microscopically after Hoechst 33258 (Sigma) staining. Cells were seeded at 10^4 cells/well in a 24-multiwell plate and treated or not with 10 μM Zol for 48 h or 100 nM staurosporine (Sigma) for 16 h, stained with 10 μg/ml Hoechst reagent for 30 min at 37°C, and then observed under UV microscopy (DMRXA; Leica, Wetzlar, Germany).

Western Blot Analysis. Zol-treated cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 5% Tris, pH 7.4, 1% Nonidet P-40, 0.25% Na deoxycholate, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Protein concentration was determined with a BCA kit (Pierce Chemical, Rockford, IL). Moreover anti-actin was used as loading control (Sigma-Aldrich, St. Louis, MO). Twenty micrograms of total cell lysate proteins was run on SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to Immobilon-P membrane (Millipore Corporation, Billerica, MA). The membrane was blotted with antibodies to P-p53 (Ser15); Bax, Bcl-2, and P-Rb (Ser795); P-Rb (Ser807/811); P-cdc2 (Tyr15); Wee1, cdc25c, and P-cdc25c (Ser216); P-chk1 (Ser345); P-ATR (Ser428); focal adhesion kinase (FAK) and P-FAK (Tyr925) (Cell Signaling Technology Inc., Danvers, MA); and $p21^{WAF1}$(BD Biosciences, San Jose, CA) in PBS, 0.05% Tween 20, and 3% bovine serum albumin. The membrane was washed and probed with the secondary antibody coupled to horseradish peroxidase. Antibody binding was visualized with the enhanced chemoluminescence system (ECL kit; Roche Molecular Biochemicals).

Caspase-1, -3, and -8 Activities. Caspase-1, -3, and -8 activities were assessed on 10 μl of total Zol-treated or not cell lysates using the kit CaspACE assay system, fluorometric (Promega, Madison, WI) following the manufacturer's recommendations. Cells treated with UV light for 30 s 24 h before harvesting were used as a positive control. Results are expressed in arbitrary units relative to the total protein content.

Cell Cycle Analysis and Mitochondrial Membrane Permeability Assay. Subconfluent cultures of OSRGA, MG63, and SAOS2 cells were incubated in the presence or absence of Zol for 48 h, trypsinized, washed twice, and incubated in PBS containing 0.12% Triton X-100, 0.12 mM EDTA, and 100 μg/ml ribonuclease A. Then, 50 μg/ml propidium iodide was added for each sample for 20 min at 4°C in the dark. Cell cycle distribution was analyzed by flow cytometry (FACScan; BD Biosciences), based on 2N and 4N DNA content.

The mitochondrial membrane potential of the cells treated with or without Zol was assessed using the MitoProbe JC-1 assay kit (Invitrogen, Carlsbad, CA) for flow cytometry after the manufacturer's recommendations. Mitochondrial depolarization is indicated by an increase in the green/red (FL1/FL2) fluorescence intensity ratio and allows detection of changes in membrane potential associated with the mitochondrial permeability transition. A mitochondrial membrane potential disrupter, carbonyl cyanine 3-chlorophenyldrazone, was used as a positive control. Fluorescence intensity of was measured by flow cytometry (FACScan; BD Biosciences).

Time-Lapse Microscopy. For time-lapse experiments, cells were seeded at 5×10^4 cells/well and cultured in six-multiwell plates in the presence or the absence of 10 μM Zol. Phase-contrast photographs (Leica) were taken every 10 min for 60 h and edited using MetaMorph software (Molecular Devices, Sunnyvale, CA). Cell divisions and apoptotic cells were then manually scored. To study the cell migration, cells were cultured in six-well plates until confluent and treated or not with 10 μM Zol for 24 h before a slit was made in the cell monolayer. Cell migration was then followed for the next 48 h.

Confocal Microscopy. For AIF and EndoG localization, cells were treated with or without Zol, fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with primary polyclonal anti-AIF antibody (diluted 1:50; Cell Signaling Technology Inc.) or polyclonal anti-EndoG antibody (diluted 1:100; ProSci Incorporated, Lausen, Switzerland) in PBS, 1% bovine serum albumin, and 0.1% Triton for 1 h, washed, and incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG; 2 mg/ml; 1:200, Invitrogen) for 45 min. Nuclei were stained with 1 μg/ml Topro3 (Invitrogen) for 30 min. Coverglass fitting was achieved with the Long Pro Kit (Invitrogen). For actin filaments detection, cells were treated with or without Zol as indicated, fixed in 4% paraformaldehyde, and stained with 0.25 μg/ml fluorescein isothiocyanate-conjugated phalloidin (Sigma). Images were collected on a TCS-SP1 confocal microscope (Leica) with 63/1.4× oil immersion lens. The digital images were visualized with a 24-bit imaging system, including TCS-NT software (Leica), and projections were generated from z-stacks.

Electron Microscopy. OSRGA cells were treated for 72 h with 10 μM Zol in plastic Petri dishes, washed with 2% NaCl, 0.15 M sodium cacodylate buffer, pH 7.2, and fixed with cacodylate-buffered 3% osmium tetroxide at 4°C for 30 min. After being rinsed, cultures were dehydrated in a graded concentration of ethanol and immersed in ethanol/Epon (1:1) for 1 h. After evaporation, cells were rinsed three times with Epon and placed at 37°C for 12 h. Epon capsules were returned onto the cell monolayer in contact with the thin, partially polymerized Epon film and placed at 60°C until polymerization was complete (3 days). After detachment of the Epon capsules containing the cell monolayers from the Petri dishes, thin sections (70–80 nm) were stained with uranyl acetate and lead citrate, and sections were examined with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan).

Results

Zoledronic Acid Induces Pro- and Antiproliferative Effects on Osteosarcoma Cells by an Integrin-Dependent Pathway. Consistent with previous results (Evdokiou et al., 2003; Heymann et al., 2005; Ory et al., 2005), Zol treatment of rat (Fig. 1A) and human (Fig. 1B) osteosarcoma cells strongly reduced their proliferation. Thus, 0.1 to 100 μM Zol decreased the viable cell number in a dose-dependent manner (IC_{50} = 1–8 μM) as revealed by the XTT assay. Similar experiments performed on primary human and rat osteoblasts showed that Zol was more potent against osteosarcoma cell lines than primary osteoblasts (IC_{50} = 10 μM) (Fig. 1, A and B). However, when lower concentrations of Zol ($1–10^4$ pM) were used, Zol exerted the opposite effect on the cell viability (Fig. 1C). The results demonstrated a 60% increase of viable rat OSRGA and human SaOS2 cells, and a 100% increase for MG63 osteosarcoma cells in the presence of 10 pM Zol after 72 h of treatment (Fig. 1C).

Because bisphosphonates modulate both the attachment of tumor cells to extracellular matrix proteins or to bone sections (Boissier et al., 1997) and the endothelial cell adhesion and migration on vitronectin (Bezzi et al., 2003), we raised the hypothesis that Zol effects may involve the integrin pathway. The integrins act as cell surface receptors, mediating cell functions (e.g., adhesion to extracellular matrix, migration, and cell death) through binding to an RGD motif (Ruoslahti, 1996; Salsmann et al., 2006). In this context, to determine whether zoledronic acid could exert its activities through integrin, the capacity of a recombinant RGD amino acid sequence to interfere with Zol activities was assessed. Thus, pretreatment of osteosarcoma cell lines with 1 mM RGD peptide prevented the inhibitory effects of 1 μM Zol previously observed on OSRGA (Fig. 1D) and SaOS2 and MG63 cell (Fig. 1, E and F) proliferation. These data demonstrate that Zol exerts differential effects on osteosarcoma cell proliferation depending on the concentrations used and that an integrin-dependent pathway is involved in Zol-induced inhibitory mechanisms.

Zoledronic Acid Induces Osteosarcoma Cell Death Independently of the p53 Status by a Caspase-Independent Mechanism but in Association with AIF/EndoG Translocation. To determine whether the inhibitory activity of Zol observed on osteosarcoma cell lines resulted from induction of cell death, we used time-lapse microscopy to monitor the apoptotic events in human and rat osteosarcoma cells treated with 10 μM Zol (Fig. 2A). The first apoptotic events occurred at an early time (5–10 h) in the Zol-treated OSRGA osteosarcoma culture, but they became significantly different compared with the control after 20 h of treatment. The observed cell death was accompanied by extensive plasma membrane blebbing characteristic of apoptotic cells (Fig. 2A, top photo). A similar phenomenon was observed on SaOS2 and MG63 human osteosarcoma cells (data not shown). To characterize the apoptotic mechanisms induced by Zol, nucleus fragmentation and caspase activations were analyzed in Zol treated-osteosarcoma cells. In contrast to 100 nM staurosporine for 16 h, which induced apoptosis associated with nucleus fragmentation, the nuclei of Zol-treated cells exhibited a characteristic kidney-like form with condensed chromatin clumps compared with control cells (Fig. 2B). Moreover, Western blot and enzymatic assays revealed no caspase-1, -3, or -8 activity in response to Zol treatment (data not shown), and the pan-caspase inhibitor Z-Vad-FMK did not inhibit the Zol-induced effects on osteosarcoma cell viability (data not shown). In the light of these data, we conclude that Zol induced atypic apoptosis of all analyzed osteosarcoma cell lines by a mechanism inde-

pendent of caspases but associated with membrane and nucleus alterations.

We next examined by Western blot the involvement of the apoptotic mitochondrial pathway in Zol-induced programmed cell death. In all osteosarcoma cell lines analyzed (OSRGA, SaOS2, and MG63), the ratio Bax/Blc2 was altered in favor of Bax, but with striking differences (Fig. 2C). Indeed, 10 μM Zol strongly up-regulated Bax expression in a time-dependent manner in OSRGA, whereas this modulation was not significant in SaOS2 and MG63 cells. Conversely, Zol decreased Bcl-2 expression in OSRGA cells, whereas this parameter remained stable in SaOS2 and MG63 cells (Fig. 2C). Zol also induced P-p53 (Ser15) in OSRGA cells, which possess a wild-type p53, in contrast to MG63 and SaOS2, which are mutated and null for p53, respectively.

To better define the role of mitochondria in Zol-induced cell death, the mitochondrial membrane potential and the mitochondrial effectors AIF and EndoG were studied. Figure 3 shows that the JC-1 mitochondrial membrane potential sensor of OSRGA, SaOS2, and MG63 was strongly increased in the presence of 10 μM Zol for 48 h as revealed by the FL1/FL2 fluorescence ratio (Fig. 3, left table). Moreover, confocal microscopy analysis revealed that 10 μM Zol treatment was followed by a time-dependent translocation of the two nucleases AIF and EndoG from a mitochondrial to a perinuclear location in the three osteosarcoma cell lines, in relation with a decrease of the Topro3 staining intensity, underlying potential DNA disruptions (Fig. 3).

Together, these results suggest that the Zol-induced atypic apoptotic in osteosarcoma cells involved apoptotic mitochondria pathways characterized by AIF/EndoG translocation independently of the p53 status.

Zoledronic Acid Induces Osteosarcoma Cell Cycle Arrest in S, G_2/M Phases Independently of the Rb Status. To determine whether the Zol-induced cell death was combined with an inhibition of cell proliferation, flow cytometry of osteosarcoma cell DNA content was performed after 24 and 48 h of 10 μM Zol treatment. Although 24 h of 10 μM Zol treatment did not modulate the cell cycle in OSRGA, SaOS2, and MG63 cells (data not shown), 48 h of Zol treatment induced a cell cycle arrest in S and G_2/M phases in all cell types (Fig. 4A). In OSRGA, SaOS2, and MG63 cells, Zol induced a cell cycle arrest in S and G_2/M phases. Indeed, the number of cells in S, G_2/M phases strongly increased from 45 to 75% for OSRGA cells, from 37 to 83% for MG63 cells, and from 38 to 60% for SaOS2 cells when treated with Zol (Fig. 4A). This observation was concomitant with a reduction of cells in G_0/G_1 phase: 51 versus 17% for OSRGA, 59 versus 6% for MG63, and 56 versus 9% for SaOS2. The cells in the apoptotic sub-G_0/G_1 peak also increased from 4 to 18 and 11% for OSRGA and MG63 cells, respectively, and from 6 to 31% for SaOS2 cells (Fig. 4A).

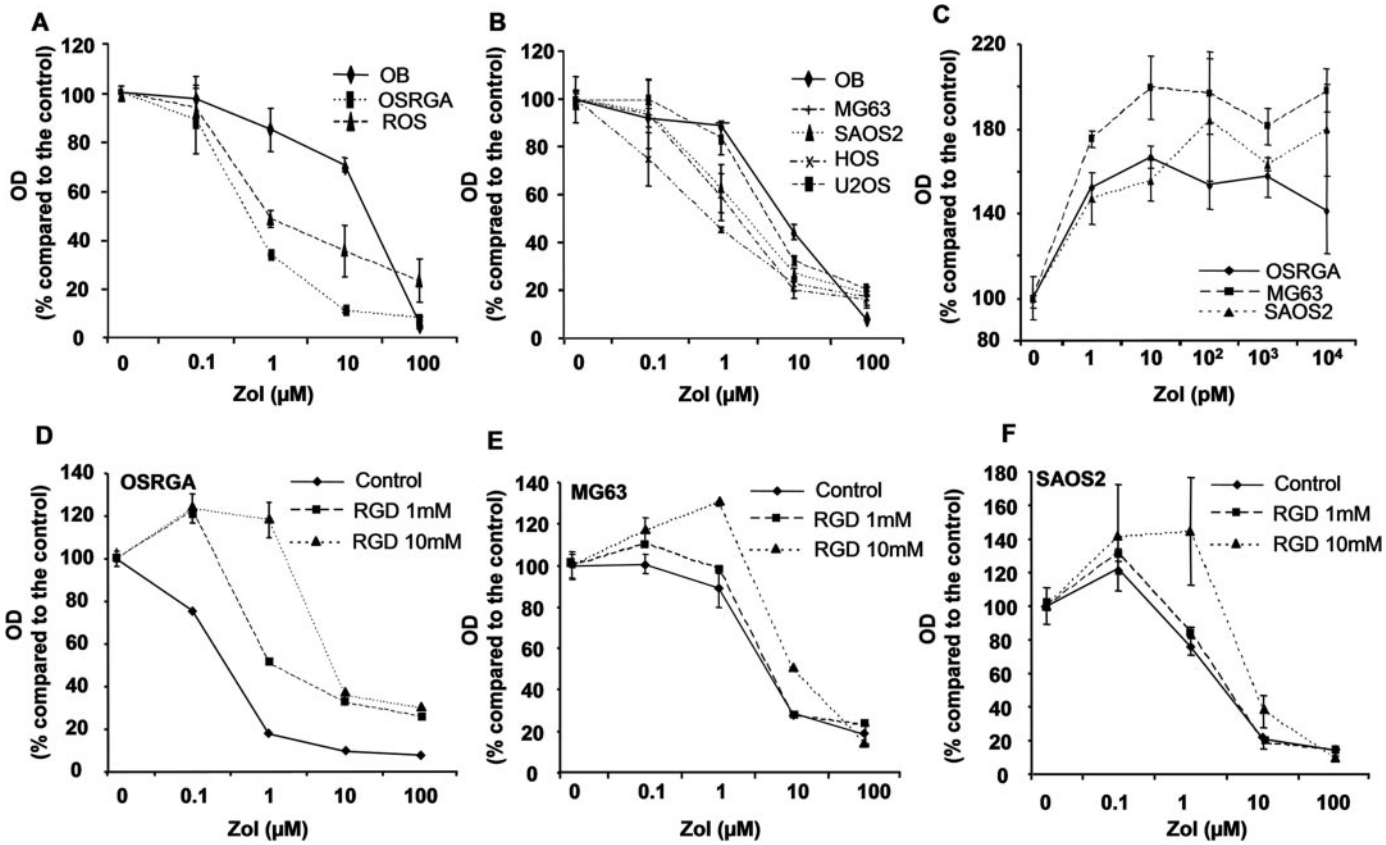

Fig. 1. Zoledronic acid treatment differentially affects the alive cell number through an integrin-dependent pathway. Several rat (A; OSRGA and ROS) and human (B; MG63, SaOS2, U2OS, and HOS) osteosarcoma cell lines were treated by increasing concentration of Zol (0.1–100 μM) for 72 h. The number of viable cells was then determined using an XTT assay and compared with primary culture of osteoblasts. C, viability assay of osteosarcoma cell lines was investigated in the presence of low concentrations of Zol (1–10^4 pM). D to F, similar experiments were performed in the presence of RGD peptide at 1 and 10 mM. Graphs represent the average values of three independent experiments performed in triplicate. Error bars represent the standard deviation.

We therefore investigated by Western blot which DNA checkpoints could be involved to delay the cell cycle progression observed in the presence of Zol. Thus, in the three osteosarcoma cell lines studied, high concentrations of Zol (10 μM) increased the inactive form of cdc2 (P-cdc2 Tyr15) after 72 h of treatment (Fig. 4B). The effect of Zol on the two regulators of cdc2 phosphorylation (cdc25c and Wee1) was further investigated. Zol promptly reduced the cdc25c phosphatase in a time-dependent manner (Fig. 4B), without affecting the phosphorylated cdc25c form, suggesting that cdc25c is predominantly present in its inactive phosphorylated form. In addition, Zol induced expression of Wee1 in MG63 an SAOS2 cells. The modulation of the effectors Wee1 and cdc25c coincided with an increase of their upstream transducers P-chk1 (Ser345) and ATR kinases (Fig. 4B). Thus, the phosphorylated form of cdc2, which is unable to interact with cyclin B, may block the cell cycle in G_2/M phase and then prevent the entry of osteosarcoma cells into mitosis, as observed by flow cytometry. In parallel with this phenomenon, 10 μM Zol strongly inhibited p21 in all osteosarcoma cell lines; transiently up-regulated Rb phosphorylation (Ser795, -807, and -81) at 24 and 48 h, respectively, in OSRGA and MG63 cells; but failed to modulate similarly P-Rb in SaOS2 cells (Fig. 4B). To understand the opposite effect of low Zol concentrations on cell proliferation, similar Western blot analyses were performed on these three osteosarcoma

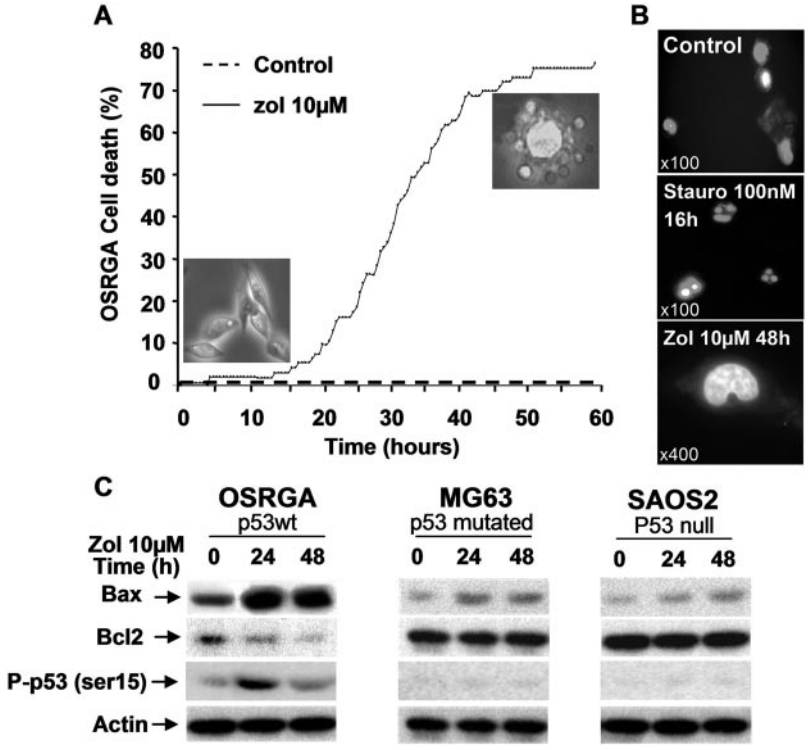

Fig. 2. Zoledronic acid induces atypic apoptosis of osteosarcoma cells independently of their p53 status. A, kinetic study of OSRGA cell death was analyzed by time-lapse microscopy in the presence or the absence of 10 μM Zol. The number of dead cells was manually scored every 10 min until 60 h. Characteristic extensive membrane blebbing of OSRGA cells in the presence (top inset) of 10 μM Zol compared with control cells (bottom inset) is shown. Zol induced similar profile on MG63 and SaOS2 cells. B, nuclear morphology was studied by Hoechst staining after Zol (10 μM; 48 h) treatment in OSRGA cell line. Staurosporine (100 nM; 16 h) was used as a positive control of nucleus fragmentation. Similar observations were obtained for MG63 and SaOS2 cells. C, implication of Bax, Bcl-2, and p53 in Zol-induced cell death was assessed by Western blot. All experiments were repeated three times, and a representative blot is shown. wt, wild type.

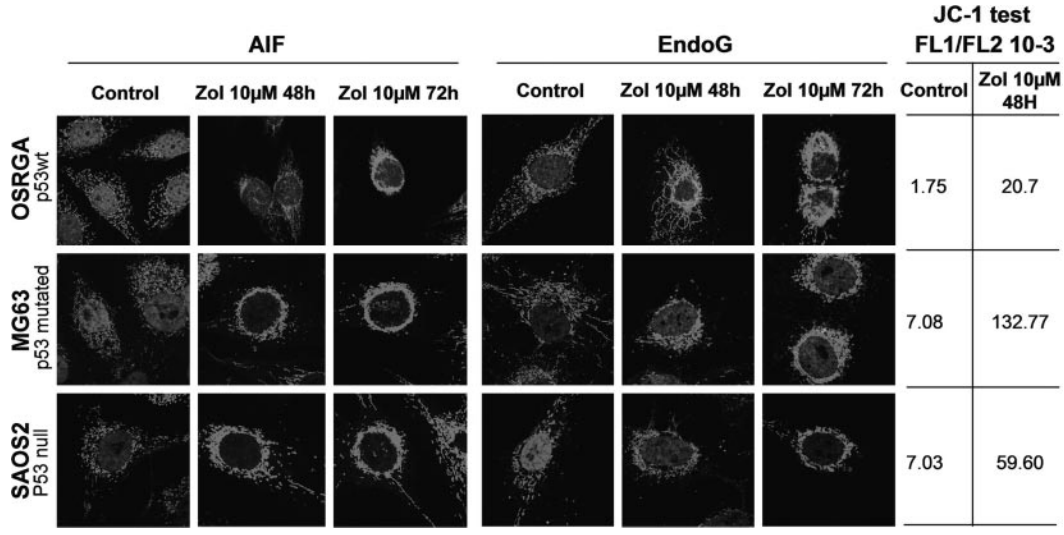

Fig. 3. Zoledronic acid stimulates mitochondrial permeability and AIF/EndoG translocation in OSRGA, MG63, and SaOS2 osteosarcoma cells. Osteosarcoma cell lines were treated by 10 μM Zol for 48 and 72 h. The mitochondrial permeability was studied by JC-1 mitochondrial membrane potential sensor as revealed by the FL1/FL2 ratio, and AIF/EndoG localization was followed by confocal microscopy using specific antibodies. Nuclei were stained by Topro3. Original magnification, 1000×.

cell lines in the presence of 10 pM and 10 nM of Zol (Fig. 4C). In contrast to high concentrations, 72-h treatment with low Zol concentrations strongly increased P-Rb in OSRGA and MG63 cells in a dose-dependent manner and not in SaOS2 cells. In the same conditions, Zol did not modulate P-cdc2 in the three cell lines. These results, in agreement with the proproliferative effects observed in the presence of low concentrations of Zol (Fig. 1C), demonstrate that low doses of Zol during 72 h of treatment had similar effects on Rb phosphorylation as high doses for shorter incubation time (24 h).

Because OSRGA and MG63 osteosarcoma cells are Rb wild type and SaOS2 is defective for Rb, our results also demonstrated that Zol blocks cell cycle and induces the cell death of osteosarcoma cells independently of their Rb status.

Zoledronic Acid Disturbs Cytoskeletal Organization and Cell Junctions and Inhibits Cell Migration. Because Zol alters osteosarcoma cell death and proliferation through an integrin-dependent pathway, we wondered whether Zol could disturb the cytoskeletal organization and cell migration. Confocal microscopic observations revealed a major disorganization of the actin stress fibers associated with membrane ruffling in the three osteosarcoma cell lines treated with 10 μM Zol for 72 h (Fig. 5A). Moreover, as shown by the time-lapse assay, 10 μM Zol totally blocked the migration of OSRGA and MG63 cells, and it strongly slowed down the migration of SaOS2 cells, which exhibit a higher proliferation rate (Fig. 5B). The effects of Zol on the cytoskeletal organization and cell migration were corroborated by the electron microscopic analysis, which showed that Zol induced striking morphological changes in cell shape, leading to the inhibition of cell interactions. Indeed, in the presence of Zol, OSRGA osteosarcoma cells were retracted, adopted a round

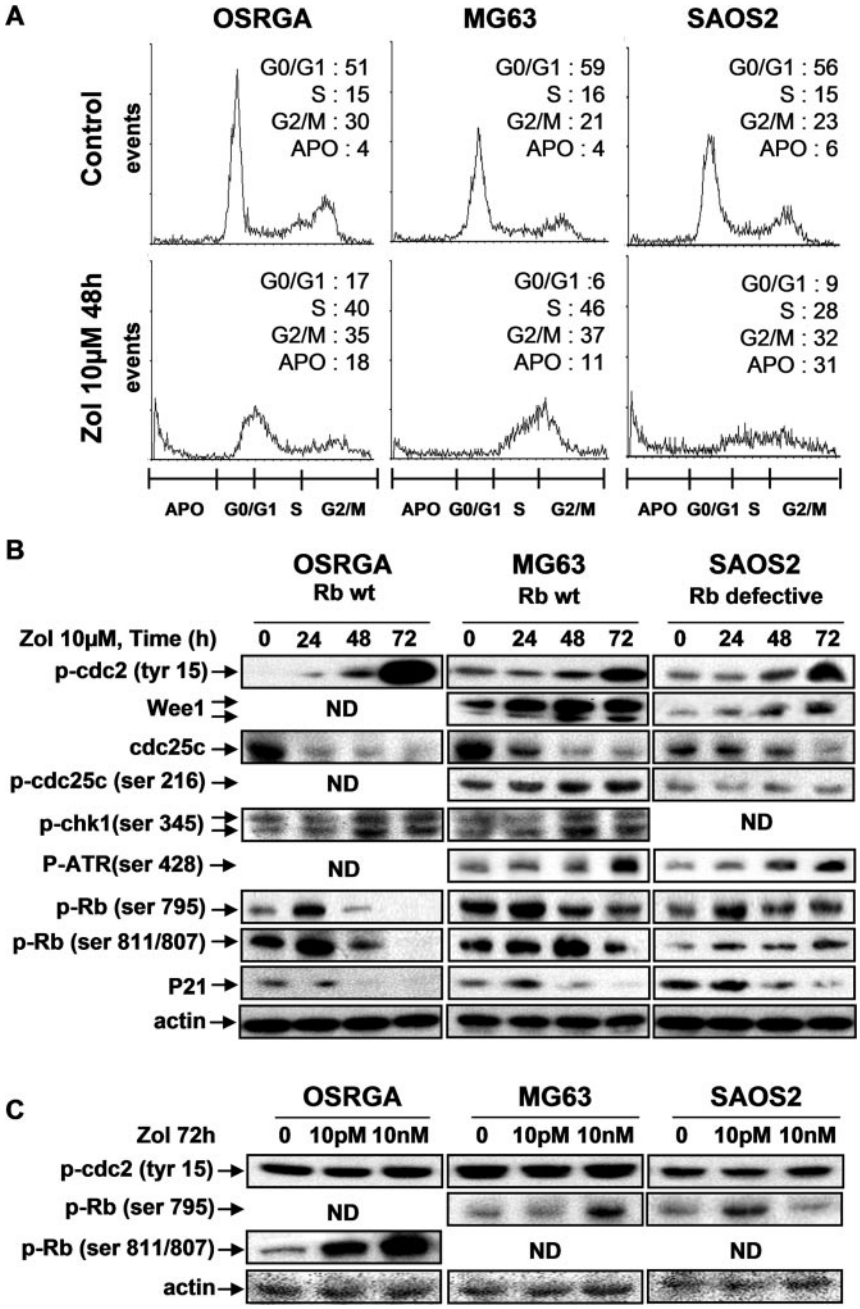

Fig. 4. Zoledronic acid blocks cell cycle of osteosarcoma cell lines in S, G_2/M phases independently of their Rb status. A, cell cycle distribution of osteosarcoma cell lines treated or not with 10 μM Zol for 48 h was analyzed by propidium iodide staining and fluorescence-activated cell sorting analysis. B, G_1/S and G_2/M DNA checkpoints were analyzed by Western blot on rat (OSRGA) and human (MG63 and SaOS2) osteosarcoma cell lines in the presence or absence of high Zol concentrations for 24, 48, and 72 h (B) or low Zol concentrations for 72 h (C). All experiments were repeated three times, and a representative blot is shown. N.D., not determined.

shape, and lost their cellular interactions. A disruption of the gap and desmosome-like junctions occurred in contrast to the control cells (Fig. 5C). These effects may be in part related to the time- and dose-dependent down-regulation of FAKs phosphorylation observed in the presence of Zol (Fig. 5D). Zol exerts the same effects on human osteosarcoma cell lines (data not shown).

GGO Partly Protects Osteosarcoma Cells from Zoledronic Acid Effects. To further determine the involvement of the mevalonate pathway in the Zol effects observed on human osteosarcoma cells, the effect of Zol on MG63 cell cycle was analyzed in the presence or the absence of 25 μM geranylgeraniol. Whereas 72 h of 10 and 25 μM Zol treatment blocked the MG63 cells in S G_2/M phases, 25 μM GGO restored a cell cycle profile similar to the control cells (Fig. 6A). The effect of 25 μM GGO was also determined by trypan blue exclusion on the MG63 cell death induced by Zol treatment. The results revealed that 25 μM GGO partly inhibited the effects of 10 and 25 μM Zol on cell death, even if the cell cycle was restored (Fig. 6, B and C). Similar data were obtained on SaOS2 human osteosarcoma and OSRGA rat osteosarcoma (data not shown).

Discussion

Despite recent improvements in surgery and the development of different regimens of multidrug chemotherapy over the past 25 years, survival of patients suffering from osteosarcoma remains around 55 to 70% after 5 years (Provisor et al., 1997). The prognosis is worse with nonextremities localization, advancing age, radioinduced osteosarcoma, and those arising from Paget's disease of bone, representing 40% of the entire osteosarcoma population. In addition, patients with metastatic osteosarcoma at the time of diagnosis have poor survival statistics (30% at 5 years). The major challenge in treating cancer relates to mutations in key genes such as p53, Rb, or proteins affecting caspase signaling carried by many tumor cells. Moreover, several mutations or inactivations of the antioncogenes p53 and Rb are detected in 50% patients suffering from osteosarcoma (Wadayama et al., 1994; Fuchs and Pritchard, 2002). In this context, p53 and Rb status become the major predictors of failure to respond to radiotherapy and chemotherapy in osteosarcomas. The poor prognosis of osteosarcoma warrants new therapeutic strategies to improve the overall rate of survival, especially in high-risk subgroups. In this context, one of the future ther-

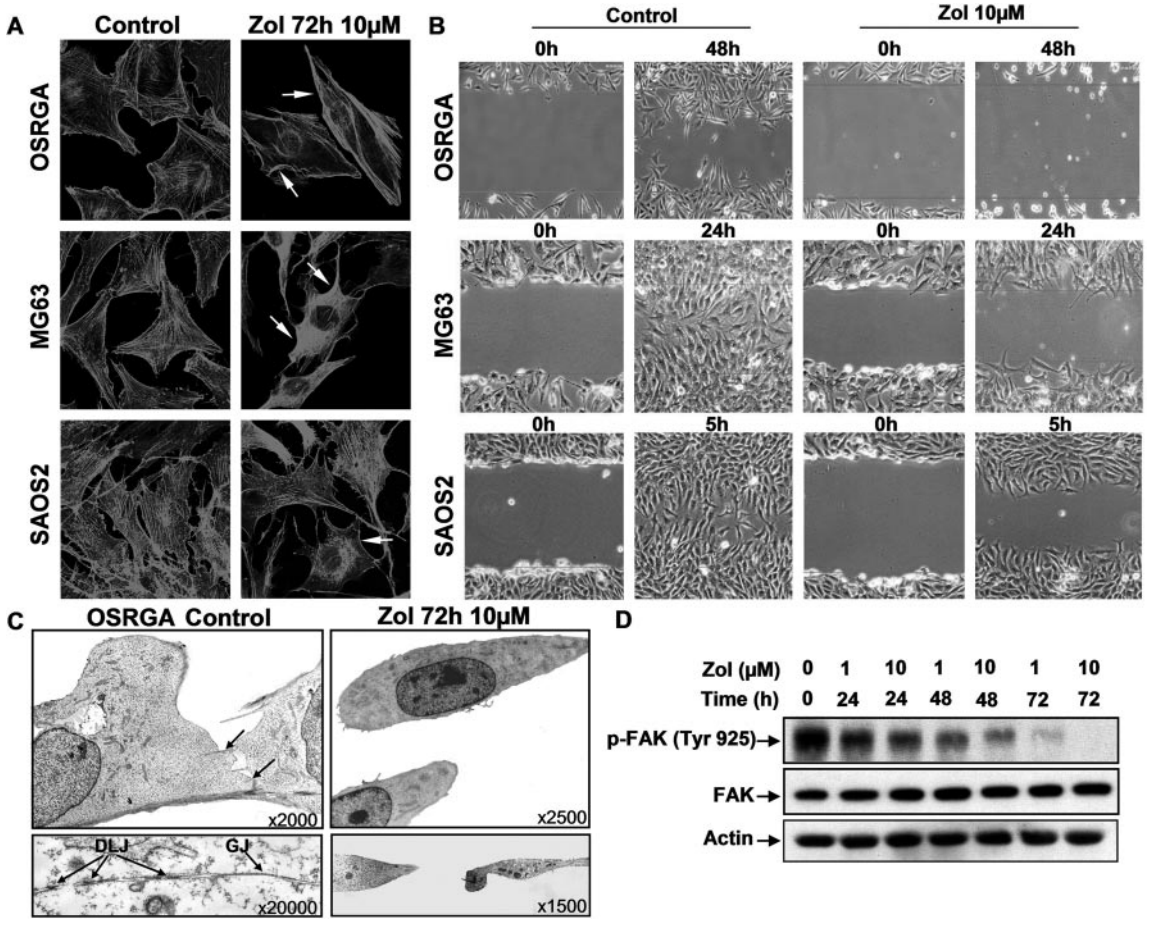

Fig. 5. Zoledronic acid disturbs cytoskeleton organization, disrupts cell junctions, and inhibits cell migration. A, Zol effects on actin stress fibers organization were observed by confocal microscopy after phalloidin staining. The actin network reorganization was associated with membrane ruffling (white arrow). Original magnification, 1000×. B, Zol effects on cell migration were observed by time-lapse microscopy. The horizontal red bars represent the limit of the slit performed on the cell monolayer at the start of the experiment. Original magnification, 100×. C, ultrastructural analysis of OSRGA cells treated or not by 10 μM Zol for 72 h. DLJ, desmosome-like junction; GJ, gap junction. D, FAK phosphorylation was assessed by Western blot on OSRGA cell line at the indicated condition of Zol treatment. All experiments were repeated three times, and a representative blot is shown.

apeutic challenges is based on therapeutic approaches bypassing p53, Rb, and caspase cascade.

Zol would exert one part of its activity on this G_1/S DNA checkpoint (Fig. 7). Indeed, Zol induces Rb phosphorylation and stimulates Rb wild-type osteosarcoma cell G_1/S progression. Rb protein belongs to the pocket protein family, which includes Rb, p107, and p130. Rb acts as a generic corepressor of the E2F family of transcription factors (Harbour and Dean, 2000). Hypophosphorylated Rb is recruited by E2F, which in turn recruits histone deacetylase, leading to active transcriptional repression (Harbour and Dean, 2000). In contrast, hyperphosphorylated Rb triggers the activity of E2F transcription factor, leading to enhanced cyclin E level. Increased cyclin E/cdk2 activity allows further Rb hyperphosphorylation, G_1 progression, and S-phase initiation (Pestell et al., 1999; Blagosklonny and Pardee, 2002). Zol exerts one part of its activity through this G_1/S DNA checkpoint (Fig. 7). Indeed, Zol induces Rb phosphorylation and stimulates Rb wild-type osteosarcoma cell G_1/S progression. The repression of p21 by Zol is in agreement with the known role of p21 to bind to and inhibit the S-phase-promoting cdk2-cyclin E complex, thus further enhancing G_1/S progression. p21 also binds to the cdk4-cyclin D complex and prevents it from phosphorylating Rb (Harper et al., 1993). Furthermore, if p21 repression reduces cell proliferation, it may also have an anticancer effect. For example, c-myc, which represses p21, sensitizes tumor cells simultaneously to apoptosis by anticancer drugs (Gartel and Radhakrishnan, 2005) However, low doses of Zol also stimulate the proliferation of the Rb-defective-SaOS2 osteosarcoma cell line, demonstrating that the Zol proproliferative activity bypasses Rb pathway. In this system, Rb phosphorylation must be discussed as a dynamic process. At early time of treatment or low Zol concentration, Rb is phosphorylated, and cells accumulate in S phase and become more sensitive to DNA damage. Because cells are not able to repair their DNA damage, they start a feedback process by inhibiting Rb phosphorylation at late time points by cyclin/cdk complex regulation (Krucher et al., 2006). Zol also stimulates proliferation of the Rb-defective SaOS2 osteosarcoma cell lines, demonstrating that the Zol proproliferative activity bypasses the Rb pathway. Indeed, the SaOS2 osteosarcoma cell line has been used frequently for the studies on the biological function of the p53 and Rb genes, because p53 is homozygously deleted and only truncated Rb protein was expressed in these cells (Huang et al., 1988). How could the Zol effect on G_1/S checkpoint be explained in the absence of

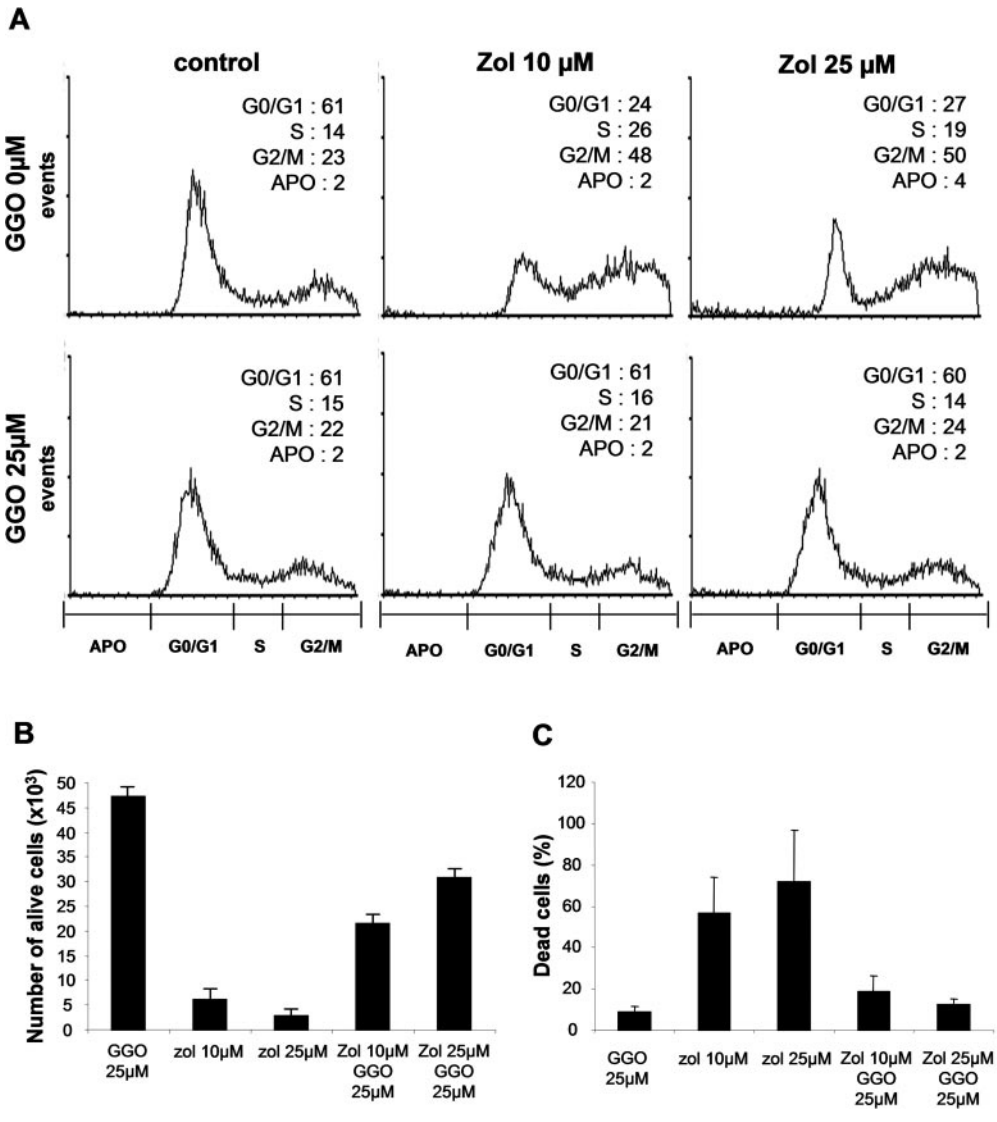

Fig. 6. GGO partly protects osteosarcoma cells from zoledronic acid effects. MG63 cells were cultured for 72 h as described above, before incubation with 10 and 25 μM Zol in the presence or the absence of 25 μM GGO. A, cell cycle distribution of MG63 cells analyzed by propidium iodide staining and fluorescence-activated cell sorting analysis. The alive (B) and dead (C) MG63 cell number (from trypsinized and floating cells) was manually scored after trypan blue exclusion.

an efficient Rb? Dimri et al. (1996) demonstrated that p21 suppresses cell growth and E2F activity in cells lacking a functional Rb protein. In this case, the p21 repression induced by Zol treatment may facilitate the S-phase entry of SaOS2 cells. More recently, Jori et al. (2005) demonstrated both specific and overlapping functions of Rb and p130 genes during the early stages of in vitro neural differentiation of marrow stromal stem cells (Jori et al., 2005), thus revealing a potential compensatory mechanism by the other Rb family genes in Rb null cells. Although p107, p130, and Rb are closely related members of the same family, they have different affinities for E2F family members, and they exhibit distinct temporal regulation during the cell cycle. Although the E2F-p130 complex is the most abundant in quiescent cells, the E2F-p107 and E2F-Rb complexes accumulate in G_1 cells but not in S, G_2, or M phases (Moberg et al., 1996). Thus, p107, which is expressed by SaOS2 cells (Gao et al., 2002), may bypass the Rb defect in these cells and could explain the observed Zol effects.

DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage (Nyberg et al., 2002). The G_2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. Depending on the DNA damage, the ATM-Chk2-cdc25c signal transduction pathway and/or the ATR-Chk1-cd25c pathway is activated to arrest the cell cycle (Sancar et al., 2004). Checkpoint kinases inhibit the entry into mitosis by down-regulating cdc25c and up-regulating Wee1, which together control Cdc2/cyclin B complex activity (Yarden et al., 2002). According to these data from the literature, high con-

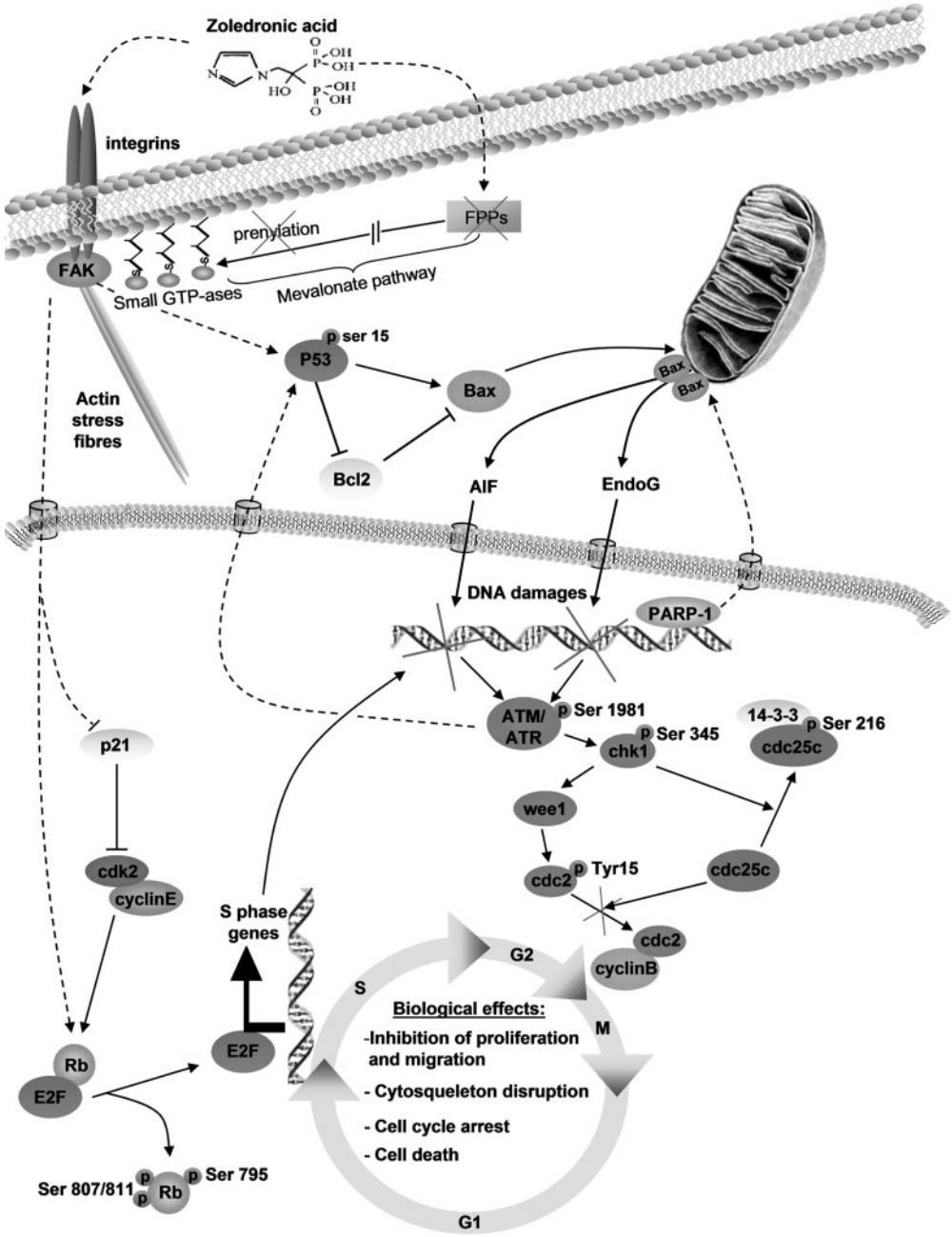

Fig. 7. Potential mechanism of action of zoledronic acid on osteosarcoma cell lines. Membrane molecules such as integrins participate in Zol activities. Low Zol concentrations facilitate the entry in S phase through a decrease of p21 and the phosphorylation of Rb and then liberate the E2F transcription factor activating the S-phase genes. High Zol concentration induced the phosphorylation of cdc2, through a decrease of cdc25c and an increase of Wee1 and the phosphorylation of chk1. P-cdc2 is then no more able to bind to the cyclin B, resulting to a cell cycle arrest in S, G_2/M phase. In tandem, Zol induces atypic apoptosis independently of caspase activation and involving the mitochondria pathway, in particular AIF and EndoG translocation, which may result in DNA damage. The Zol affects the cell cycle and cell death of osteosarcoma cell lines by an integrin-dependent mechanism but independently of the p53 and Rb status of the cells.

centrations of Zol strongly inhibit cell proliferation and induce a cell cycle arrest in S, G_2/M phase, presumably via the control of the intra-S and G_2/M checkpoint (Fig. 7). Indeed, this intra-S and G_2/M checkpoint delays transiently cell cycle progression through S phase to allow the repair of DNA damage. If the DNA damage is not repaired during this period, the intra-S and G_2/M checkpoint should block the cell cycle later in G_2/M phase to avoid "catastrophic mitosis" (Bartek et al., 2004) as observed in the presence of Zol, hypothesize in opposition with Kubista et al. (2006). Moreover, this checkpoint does not require p53, which is the main target of the sustained G_1/S checkpoint (Bartek et al., 2004).

Because these control checkpoints are usually activated in response to DNA damage, two hypotheses can be proposed to understand Zol effects on osteosarcoma cell lines. First, the Zol influence on the G_1/S checkpoint could facilitate and accelerate the entry of osteosarcoma cells into S phase (genetically, the most vulnerable period). The accelerated transcription of S-phase genes could result in numerous DNA damage at multiple points after transcription errors, resulting, for example, from base pair mismatches or limiting dNTP pools. Indeed, intracellular dNTP pools occur as a common feature involved in DNA repair, because limiting dNTP pools enhance damage sensitivity (Zhao et al., 1998). The DNA damage caused by such phenomenon is responsible for the activation of the checkpoint-specific sensors, in particular ataxia-telangiectasia, mutated and/or ATR members of the phosphoinositide 3-kinase-like kinase family (Helt et al., 2005). These sensors are key molecules in the G_2/M DNA checkpoint, as underlined by the consequences induced by their mutations and the induced cancer predispositions (Shiloh, 1997). Second, high concentrations of Zol increase mitochondrial permeability and AIF/EndoG translocation, which may contribute to apoptotic nuclear DNA damage in a caspase-independent way, as reported in other models (Cregan et al., 2004). Such DNA damage could activate the intra-S DNA checkpoint, which slows down the S-phase progression to allow DNA repair and the G_2/M cell cycle arrest to prevent cell mitosis in the presence of DNA damage. Moreover, nuclear and mitochondrial conversations in cell death have recently been pointed out (Hong et al., 2004). Poly(ADP-ribose) polymerase 1 (PARP1) is emerging as an important activator of caspase-independent cell death, and the overactivation of PARP1 or/and DNA damage initiates a nuclear signal that propagates to mitochondria and triggers the release of AIF. Thus, a vicious cycle takes place between mitochondria and nucleus via AIF and potentially PARP1 independently of p53 (Fig. 7). Similar p53-independent effects of Zol were also described on leukemia and colorectal carcinoma cell lines (Kuroda et al., 2004).

Zol also modulates strongly the mevalonate pathway. Indeed, the mevalonate pathway is responsible for the production of cholesterol and isoprenoid lipids such as isopentenyl diphosphate, FPP, and geranylgeranyl diphosphate. FPP and geranylgeranyl diphosphate are required for the posttranslational lipid modification (prenylation) of small GTPases such as Ras, Rho, and Rac. Prenylation involves the transfer of a farnesyl or geranylgeranyl lipid group onto a cysteine residue in characteristic carboxyl-terminal motifs, giving rise to farnesylated and geranylgeranylated proteins. To our knowledge, the majority of the prenylated proteins identified are small GTPases (preferentially geranylgeranylated), which are important signaling proteins regulating a variety of cell processes such as the control of cell morphology, integrin signaling, trafficking of endosomes, and apoptosis. Prenylation is then required for the correct function of these proteins because the lipid prenyl group allows the anchorage of the proteins in cell membranes and also may participate in protein-protein interactions (Rogers et al., 2000). Therefore, the inhibition of the mevalonate pathway by nitrogen-containing bisphosphonates and consequently the loss of prenylation of small GTPases such as Rho, Rac, cdc42, and Rab could account for the various effects observed on osteosarcoma cells (disruption of the actin cytoskeleton, disrupted intracellular signaling by integrins, and induction of cell death).

The anti-oncogene p53 plays a key role in the control of cell proliferation and/or of programmed cell death (Moll et al., 2005). Pivotal to the tumor-suppressor activity of p53 is its ability to activate apoptosis via multiple caspase-dependent or caspase-independent pathways (Lane, 1992). Phosphorylation of p53 represses Bcl-2 expression and up-modulates Bax expression, resulting in apoptosis via the mitochondrial pathway (Park et al., 2005), as observed in Zol-treated OS-RGA cells. However, the mechanism by which p53 is activated remains to be elucidated. For example, Zol-induced DNA damage may maintain p53 activation (Tibbetts et al., 1999). Furthermore, the FAKs may also participate in p53 activation. Indeed, FAK is a tyrosine kinase considered as a central molecule in integrin-mediated signaling, involved in cellular motility and protection against apoptosis (Mukhopadhyay et al., 2005). This characteristic is in agreement with the observed Zol effects, including an integrin-dependent mechanism, mediating a decreased of P-FAK, decreased motility and apoptosis. More recently, a direct interaction of the N-terminal domain of FAK with the N-terminal transactivation domain of p53 has been demonstrated previously (Goluboskaya et al., 2005), strengthening the potential role of p53 and FAK in Zol-induced cell death in wild-type p53 osteosarcoma cells. However, Zol induced the cell death of p53-mutated MG63 and of null p53 SaOS2 cells, thereby suggesting that Zol is able to bypass the p53 pathway to activate cell death mechanisms in osteosarcoma cells. In this case, FAK may represent a privileged place in the Zol mechanism of action. Indeed, Boissier et al. (1997) demonstrated that BPs inhibit carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrix, suggesting that BPs exert their activities via adhesion molecules. This observation has been more recently confirmed in endothelial cells (Bezzi et al., 2003). These authors demonstrated that Zol sensitizes endothelial cells to tumor necrosis factor-induced, caspase-independent programmed cell death and identified the FAK-protein kinase B/Akt pathway as a novel Zol target. Adhesion molecules must be considered as key factors for Zol activities, but other investigations are needed to determine whether FAK is the first target involved in Zol effects, via membranous integrins, or whether the effects are the consequence of Zol on cell death.

We previously demonstrated that Zol enhances osteosarcoma regression in vivo in rodent models (Heymann et al., 2005; Ory et al., 2005) and slows down rat primary chondrosarcoma development (Gouin et al., 2006). Skerjanec et al. (2003) showed that the peak levels of ZOL detected in plasma of patients suffering from cancer is around 1 μM, which is in

agreement with the IC_{50} of ZOL measured on osteosarcoma cells in the present study. Furthermore, because of its high affinity for bone matrix, the local concentration of Zol in this tissue is certainly higher than its plasma levels, thereby strengthening an in vivo potential effect of ZOL even at a low concentration. Taken together, these data demonstrate that treatment of osteosarcoma cells with an *N*-BP strongly inhibits cell proliferation through the induction of cell death via AIF and EndoG translocation and the cell cycle arrest in S, G_2/M phase independently of p53, Rb, and caspases. These observations open a new area in the field of therapeutic combinations for the treatment of osteosarcoma.

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